Galloyl cyclic-imide derivative CH1104I inhibits tumor invasion through suppressing matrix metalloproteinase activity

Ming-Hui Chen[a], Shu-Xiang Cui[b], Yan-Na Cheng[a], Li-Rui Sun[a], Qian-Bin Li[a], Wen-Fang Xu[a], Stephen G. Ward[c], Wei Tang[a,d] and Xian-Jun Qu[a]

Matrix metalloproteinase (MMP)-2 and MMP-9 have been associated with the ability of tumor cells to metastasize because of their capacity to degrade type IV collagen, the main component of basement membrane, and to their elevated expression in malignant tumors. (*S*)-methyl 6-(benzyloxycarbonylamino)-2-(2-((*S*)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido) piperidin-1-yl) acetamido) hexanoate (CH1104I) is a galloyl cyclic-imide derivative designed to fit and extend into the S1′ active pocket of MMP-2 and MMP-9. We aimed to evaluate the efficacy of CH1104I as a candidate compound for antiinvasion and antimetastasis of tumor cells. CH1104I significantly blocked gelatinase activity as evidenced by a decrease in the degradation of succinylated gelatin. Gelatin zymography analysis showed that the compound (7–210 μmol/l) inhibited the activity of MMP-2 and MMP-9 produced by human ovarian carcinoma SKOV3 cells. Inhibition of MMP-2 and MMP-9 expression was also observed using the assays of immunocytochemical staining and western blot analysis. The results showed that CH1104I suppressed the expression of zymogens and active MMP-2 and MMP-9. The effects of CH1104I on the invasion and migration of SKOV3 cells were then measured. Both the trans-well motility assay and wound scratch assay indicated that CH1104I was very effective for the antiinvasion and antimigration of SKOV3 cells. Furthermore, the Lewis lung carcinoma model was used to evaluate the efficacy of CH1104I *in vivo*. A significant inhibition of pulmonary metastasis of carcinoma cells was observed in CH1104I-administrated mice (25–100 mg/kg). These results suggest that CH1104I is a potential MMP-2 and MMP-9 inhibitor that may effectively suppress tumor invasion and metastasis. *Anti-Cancer Drugs* 19:957–965 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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[a]Department of Pharmacology, School of Pharmaceutical Sciences, Shandong University, [b]Department of Pharmacology, Institute of Materia Medica, Shandong Academy of Medical Sciences, China, [c]Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, UK and [d]Department of Surgery, Hepato-Biliary-Pancreatic Surgery Division, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Correspondence to Professor Xian-jun Qu, School of Pharmaceutical Sciences, Shandong University, Jinan 250012, China
Tel/fax: +86 531 88382490;
e-mail: qxj@sdu.edu.cn

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Introduction

Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, play a crucial role in extracellular matrix degradation associated with cancer cell invasion, metastasis, and angiogenesis [1–3]. Among members of the MMPs family, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are particularly upregulated in malignant tumors and contribute to the invasion and metastatic spread of cancer cells by degrading type IV collagen, a major component of the basement membrane (BM) [4–6]. Thus, MMP-2 and MMP-9 have been targeted in the development of drugs against tumor invasion and metastasis.

In an earlier studies, we constructed a series of galloyl cyclic-imide derivatives that were designed based on the structures of L-iso-glutamine derivatives, to fit the S1′ active pocket in molecule of MMP-2 and MMP-9 [7,8]. The target compounds were then evaluated for inhibitory activity toward MMP-2 and amino-peptidase N *in vitro*. Most of the compounds seem to be selective to MMP-2 [7]. (*S*)-methyl 6-(benzyloxycarbonylamino)-2-(2-((*S*)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido) piperidin-1-yl) acetamido) hexanoate (CH1104I) (mentioned as 4i in Li's paper) is one of these galloyl cyclic-imide derivatives that display a substantial potential inhibitory effect on MMP-2 [7]. Structure–activity relationship studies showed that the inhibitory effect of CH1104I on MMP-2 was correlated to its bulky side chain, which interact with the hydrophobic domains in enzymes [7]. In this report, we describe the inhibitory effects of CH1104I on the expression of MMP-2 and MMP-9 in human ovarian carcinoma cell line SKOV3. We also describe suppression by CH1104I of in-vitro invasion and in-vivo metastasis of tumor cells.

Materials and methods

Chemicals

CH1104I (Fig. 1) was synthesized from readily available dicarboxylic acid through a sequence reaction including

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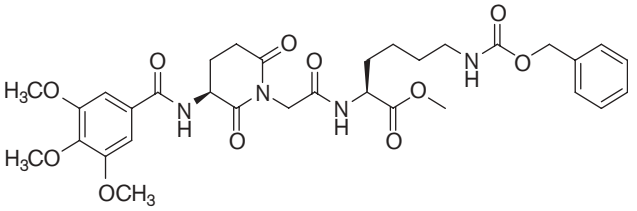
Fig. 1



Structure of (*S*)-methyl 6-(benzyloxycarbonylamino)-2-(2-((*S*)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido) piperidin-1-yl) acetamido) hexanoate (CH1104I).

dehydration and condensation with glycine and then with various amino acids or NH_2OH to give target compound [7]. The compound was dissolved in dimethylsulfoxide for in-vitro assay and in 5% amylum for in-vivo study.

Cell line and cell culture

The human ovarian carcinoma SKOV3 cell line was obtained from Shanghai Cell Bank, the Institute of Cell Biology, China National Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin–streptomycin (100 IU/ml–100 μg/ml), 2 mmol/l glutamine, and 10 mmol/l Hepes buffer at 37°C in a humid atmosphere (5% CO_2 – 95% air) and were harvested after brief incubation in 0.02% EDTA-PBS.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cells (5000 per well) seeded in 96-well plates for 12 h were treated with different doses of CH1104I for the required time period. The medium was then removed and the wells were washed with PBS. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 μl MTT (5 mg/ml, Sigma, St Louis, Missouri, USA) for 4 h. Light absorbance of the solution was measured at 570 nm on a THERMOmax microplate reader (Molecular Devices, Sunnyvale, California, USA). Triplicate experiments with triplicate samples were performed [9].

Succinylated gelatinase assay

The assay was performed in 96-well flat-bottom microtiter plates [10]. The reaction mixture (total 150 μl) contained 12.5 μl of gelatinase (30 μg/ml, Sigma), 55 μl (20 μg) of succinylated gelatin (Sigma) and, 15 μl (50 μg) of *p*-aminophenylmercuric acetate (Sigma) as well as different doses of CH1104I. The reactions were carried out at 37°C for 30 min. Fifty microliters of 0.03% trinitrobenzene sulfonic acid (Sigma) was then added to the reaction mixture and allowed to incubate at room temperature (RT) for 20 min. Absorbance at 450 nm of each reaction was determined using a THERMOmax microplate reader (Molecular Devices). The inhibitory rate (%) was evaluated by comparing the relative activity in the presence and absence of CH1104I.

Gelatin zymography

The gelatinolytic activities of MMP-2 and MMP-9 in the conditioning culture medium were assayed by electrophoresis on 10% polyacrylamide gels containing 1 mg/ml gelatin [10,11]. In brief, cells (80% confluent in 24-well plates) were washed twice with PBS and treated with different doses of CH1104I in 2 ml of serum-free medium for 24 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Ten microliters of the supernatant were subjected to electrophoresis on 10% SDS–polyacrylamide gel electrophoresis copolymerized with 1 mg/ml gelatin as a substrate. After electrophoresis was completed, the gel was washed with 2% Triton X-100 solution to remove SDS and incubated in an activation buffer (50 mmol/l Tris, 5 mmol/l $CaCl_2$, 0.5 μmol/l $ZnCl_2$, pH 7.4) for 20 h at 37°C. Gels were then stained with 0.05% Coomassie brilliant blue R-250 (Sigma) and destained in acetic acid. Nonstaining regions of the gel corresponding to gelatinase activity were quantified by densitometry using an Electrophoresis Image Analysis System (FR980, Furi Company, Shanghai, China).

Immunocytochemical staining

Cells grown on the glass slips were fixed with 4% formaldehyde in PBS for 15 min, and then permeabilized by treating with 0.1% Triton X-100 in PBS for 8 min at RT. After treating the cells with a blocking solution (5% BSA, 0.1% gelatin, and 5% rabbit or goat normal serum in PBS) for 30 min at RT, rabbit anti-MMP-2 antibody (1 : 100) or goat anti-MMP-9 antibody (1 : 100) was added and incubated for 1 h at RT. The cells were washed and incubated with biotinylated secondary antibody for 1 h at 37°C. After washing with PBS, the bound antibody was then tested for by the biotin–streptavidin–peroxidase complex method using a commercial kit (SABC kit; Boster, China). The slips were rinsed and stained by hematoxylin and eosin [12,13].

Western blot analysis

The expressions of MMP-2 and MMP-9 were also evaluated using western blot analysis [14,15]. Cells (1×10^5 per well) seeded in 6-well plates were treated with different doses of CH1104I for 24 h. The medium was removed and the cells were washed with PBS. Cells were then lysed in 100 μl of lysis buffer through three freeze–thaw cycles between −80°C and 37°C. Total protein was determined using the Bradford method [16]. Equal amounts of protein in the cell extracts were fractionated by 10% SDS–polyacrylamide gel electrophoresis and then electrotransferred onto nitrocellulose membranes. After blocking with Tris-buffered saline and 0.1% Tween-20 buffer (20 mmol/l) containing 5% nonfat dry milk for 1.5 h at RT, the membranes were incubated with polyclonal anti-MMP-2 (against amino acids 1–76 of

MMP-2 of human origin, Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) or polyclonal anti-MMP-9 (against amino acids 459–587 of MMP-9 of human origin, Santa Cruz Biotechnology) for 2 h, which was followed by washing three times and reaction with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The proteins were then detected by chemiluminescence agents (ECL, Pierce, USA).

Invasion assay *in vitro*

A 24-well trans-well chamber (Corning, New York, USA) was used to evaluate the motility and invasive ability of SKOV3 cells *in vitro* [14]. The upper surface of polycarbonate filters with 8-μm pores was coated with 100 μg of Matrigel (Sigma). SKOV3 cells were preincubated with different doses of CH1104I or 1% BSA (negative control) for 12 h at 37°C in a CO_2 incubator and then detached and resuspended in serum-free RPMI-1640. A suspension of cells (1×10^5 cells/100 μl) was placed in the upper chambers. The lower chambers were filled with 500 μl of the RPMI-1640 medium. After 24 h of incubation at 37°C under optimal conditions, the filters were fixed with 10% buffered formalin and stained with hematoxylin. Cells that had invaded through the Matrigel and reached the lower surface of filter were quantified by counting the number of cells that migrated in five random microscopic fields per filter at a magnification of $\times 400$.

Scratch assay

Scratch assay was performed by plating cells in a 6-well culture dish. After SKOV3 cells were allowed to attach and reach 80% confluence, a scratch (1 mm) was made through the culture dish with a sterile plastic 200-μl micropipette tip to generate one homogeneous wound along each well. After wounding, the peeled off cells were removed with two PBS washes. Cells were further incubated without or with different concentrations of CH1104I for 24 and 48 h and the wound widths were measured under the microscope using an ocular grid [17]. Three wounds were sampled for each specimen.

Metastasis assay *in vivo*

The antimetastasis activity of CH1104I was assessed using a Lewis lung carcinoma (LLC) model *in vivo* [18]. C57/BL6 mice, 5–6 weeks of age, were purchased from the Animal Experiment Central of Beijing, China. The research protocol was approved in accordance with the institutional guidelines of the Animal Care and Use Committee at Shandong University, China. Animals were housed under pathogen-free conditions. C57/BL6 mice arranged in groups were injected in the right footpad with 5×10^5 tumor cells in 50 μl of PBS. After 24 h, the mice were administered CH1104I via oral gavage at doses of 0, 25, 50 and 100 mg/kg in 0.5 ml of 5% amylum. Carboxylates (MMPs inhibitors, 100 mg/kg) were given orally as the positive control [19]. After 7 days, when the footpad tumors reached between 6 and 8 mm in mean diameter, the tumor-bearing legs were amputated. Administrations were performed 6 days per week for four consecutive weeks. All the surviving mice were then killed and the lungs with tumor nodes were placed in Bouin's solution. The number of the metastatic nodes on the lung surface was counted [18].

Statistical analysis

Statistical significance was determined by the Student's two-tailed *t*-test. The limit of statistical significance was a *P* value of less than 0.05.

Results

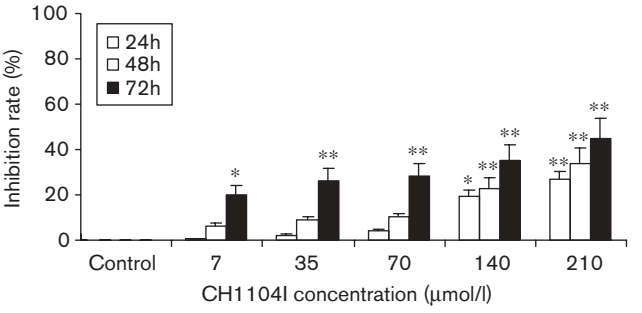
Proliferation of SKOV3 cells exposed to CH1104I *in vitro*

The growth inhibition of SKOV3 cells was examined in the presence of CH1104I. SKOV3 cells were treated with CH1104I (7, 35, 70, 140, and 210 μmol/l) for up to 72 h and then the rates of cell growth inhibition were evaluated based on the viable cell number as estimated by MTT assay. As shown in Fig. 2, incubation with CH1104I weakly prevented SKOV3 cells proliferation, although significant differences were observed in higher doses (140 and 210 μmol/l). The maximum inhibition rate was 44.9% for 210 μmol/l of CH1104I at 72 h incubation. The growth inhibition occurred in the absence of evident cytotoxicity, as verified by staining for viability using Trypan blue (data not shown).

Inhibitory effect on gelatinase activity

We evaluated the effect of CH1104I on the activity of gelatinase in hydrolysis of succinylated gelatin. As shown in Fig. 3, the degradation of succinylated gelatin by gelatinase was significantly inhibited by CH1104I in a

Fig. 2



Inhibitory effect of (*S*)-methyl 6-(benzyloxycarbonylamino)-2-(2-((*S*)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido) piperidin-1-yl) acetamido) hexanoate (CH1104I) on SKOV3 cell growth. Cells were cultured without (control) or with CH1104I (7, 35, 70, 140, and 210 μmol/l). Cell growth was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at various culture times (24–72 h). The inhibition rate at individual time point was calculated as a mean percentage of that of control at the same time point. The bars indicate means ± SD ($n=3$). *$P<0.05$; **$P<0.01$ versus control.

dose-dependent manner. The half maximal inhibitory concentration was 0.62 ± 0.14 μmol/l.

Decrease of matrix metalloproteinase-2 and matrix metalloproteinase-9 secretion and activity in SKOV3 cells

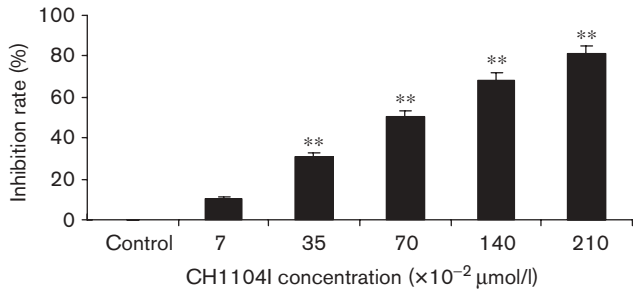
Gelatin zymography was then used to evaluate the secretion and activity of MMP-2 and MMP-9 produced by SKOV3 cells in the presence of CH1104I. As shown in Fig. 4, the activity of MMP-2 and MMP-9 in the supernatants of the cultured cells was reduced in a dose-dependent manner by treatment with CH1104I. The inhibition rates by 7, 35, 70, 140, and 210 μmol/l of CH1104I were 12.7, 16.9, 30.6, 63.9, and 82.5%, respectively, for MMP-9 and 14.8, 25.8, 26.9, 40.6, and 53.7%, respectively, for MMP-2.

Inhibition of matrix metalloproteinase-2 and matrix metalloproteinase-9 expression in SKOV3 cells

The effect of CH1104I on the expression of MMP-2 and MMP-9 in SKOV3 cells was then evaluated using immunocytochemical staining. As shown in Fig. 5, the expressions of MMP-2 and MMP-9 were chiefly observed in the nucleus of SKOV3 cells and the levels of expression apparently decreased as a result of exposure to CH1104I for 24 h. At the concentrations of 7, 35, 70, 140, and 210 μmol/l, the rates decreased to 8.7, 12.4, 25.5, 43.7, and 57.6% (Fig. 5b–f), respectively, for MMP-2 positive cells and 11.1, 15.6, 34.4, 46.3, and 68.7% (Fig. 6b–f), respectively, for MMP-9 positive cells.

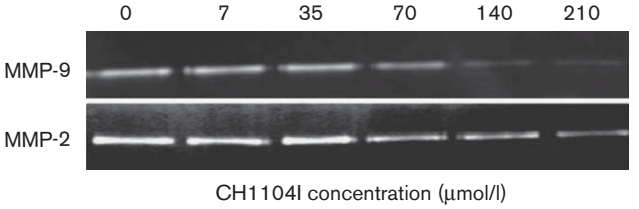
The expressions of MMP-2 and MMP-9 in SKOV3 cells were also evaluated using western blot analysis. As shown in Fig. 7, pro and active MMP-2 were measured in the gel around positions at molecular weights of 72 and 63 kDa in SKOV3 cells. The level of MMP-2 was decreased in a dose-dependent manner after 24 h incubation with CH1104I. The inhibition rates by 7, 35, 70, 140, and 210 μmol/l of CH1104I were 16.7, 44.2, 67.0, 78.3, and 93.7%, respectively, for pro-MMP-2 and 37.7, 42.0, 64.9, 78.6, and 94.9%, respectively, for active MMP-2. MMP-9 was measured around positions at molecular weight of 92 kDa in the same gel (Fig. 7). The expression was inhibited by 9.5, 20.1, 37.2, 55.6, and 72.4%, respectively, for the same concentrations as described above.

Fig. 3



Rate of gelatinase activity inhibition by (*S*)-methyl 6-(benzyloxycarbonylamino)-2-(2-((*S*)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido) piperidin-1-yl) acetamido) hexanoate (CH1104I). Proteolysis of succinylated gelatin by gelatinases was evaluated in the presence of different concentrations of CH1104I for 30 min. After the enzyme reaction, 0.03% trinitrobenzene sulfonic acid was added to each sample, which was allowed to stand for 20 min at room temperature, and then absorbance at 450 nm was measured. The inhibitory rate was evaluated by comparing the relative activity in the presence and absence (control) of CH1104I. The bars indicate means ± SD ($n=3$). *$P<0.05$, **$P<0.01$ versus control.

Fig. 4



Proteolytic activity of matrix metalloproteinase (MMP)-2 and MMP-9 in SKOV3 cells after treatment with (*S*)-methyl 6-(benzyloxycarbonylamino)-2-(2-((*S*)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido) piperidin-1-yl) acetamido) hexanoate (CH1104I). Cells were exposed to CH1104I (0, 7, 35, 70, 140 and 210 μmol/l) for 24 h. The activities of MMP-2 and MMP-9 in the medium were detected as two destained bands by SDS-polyacrylamide gel electrophoresis gelatin zymography. The inhibitory rate was evaluated by comparing the densitometry in the presence and absence (control) of CH1104I.

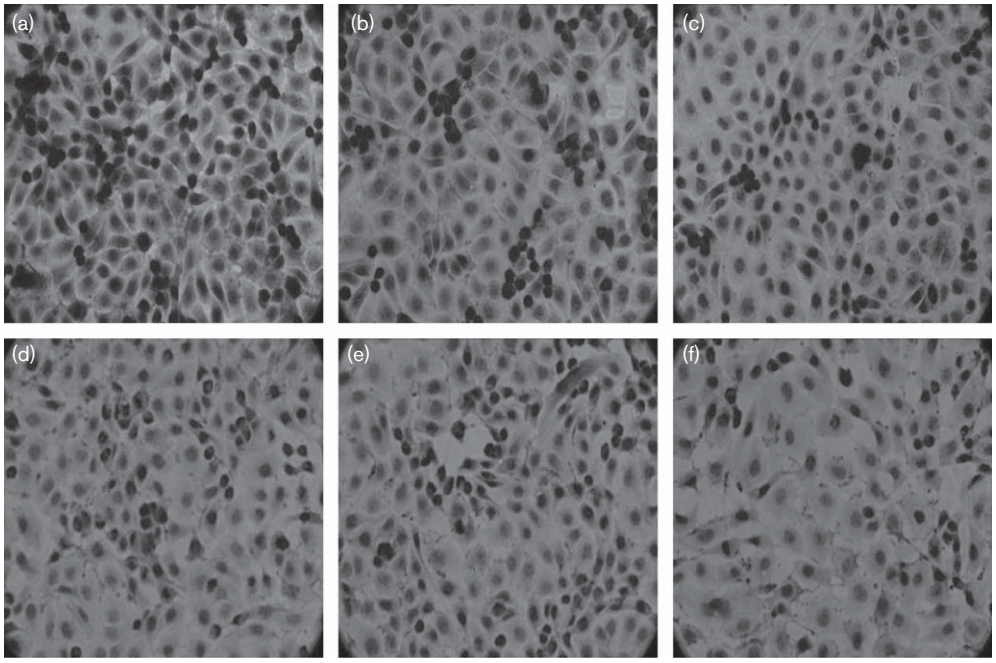
Inhibition of SKOV3 cells invasion

The activity of invasion of SKOV3 cells in the presence of CH1104I was examined. SKOV3 cells displayed a high invasive ability to penetrate Matrigel in the absence of CH1104I (Fig. 8a). The invasive potential was significantly diminished in a dose-dependent manner by 12 h pretreatment with CH1104I. At concentrations of 7, 35, 70, 140, and 210 μmol/l, the number of cells penetrating the Matrigel-coated polycarbonate filters was inhibited by 22.7, 30.8, 55.8, 70.4, and 83.5%, respectively (Fig. 8b–f).

Inhibition of SKOV3 cell migration

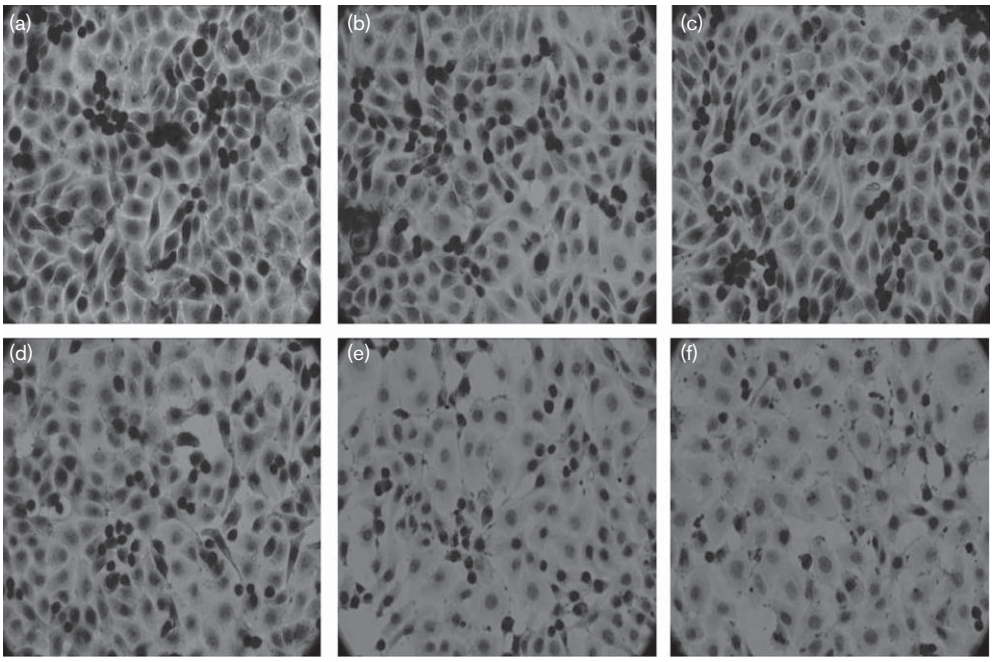
Using the scratch-wound assay, an inhibition of SKOV3 cell migration was observed in the presence of CH1104I. Fig. 9a showed the continuous rapid cell migration for up to 48 h, where a highly confluent (90–100%) monolayer region gradually migrated into the cell-free 'scratch' region. The spontaneous migration distances at 24 and 48 h of incubation were 740 and 840 μm, respectively. The activity of cell migration was markedly reduced in the presence of CH1104I. As shown in Fig. 9b, the migration distances of SKOV3 cell exposure to 7, 35, 70, 140, and 210 μmol/l of CH1104I were 400 ± 69.5, 250.6 ± 41.7, 150.2 ± 39.6, 120.7 ± 23.4, and 80.5 ± 6.3 μm, respectively, for 24 h of incubation and

Fig. 5



The immunocytochemical staining of matrix metalloproteinase-2 in SKOV3 cells. Cells grown on the glass slip were treated with different concentrations of (*S*)-methyl 6-(benzyloxycarbonylamino)-2-(2-((*S*)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido) piperidin-1-yl) acetamido) hexanoate (CH1104I) for 24 h and the immunocytochemical staining was performed as described in Material and methods. Without (a) or with 7 μmol/l (b), 35 μmol/l (c), 70 μmol/l (d), 140 μmol/l (e), and 210 μmol/l (f) of CH1104I, respectively (magnification, × 400).

Fig. 6



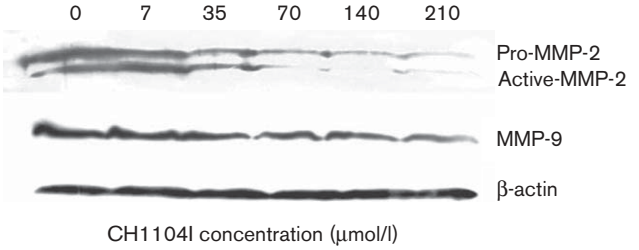
The immunocytochemical staining of matrix metalloproteinase-9 in SKOV3 cells. After treatment with (*S*)-methyl 6-(benzyloxycarbonylamino)-2-(2-((*S*)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido) piperidin-1-yl) acetamido) hexanoate (CH1104I) for 24 h, the immunocytochemical staining was performed as described in Fig. 5. Without (a) or with 7 μmol/l (b), 35 μmol/l (c), 70 μmol/l (d), 140 μmol/l (e), 210 μmol/l, and (f) of CH1104I, respectively (magnification, × 400).

460.8 ± 81.4, 390.5 ± 38.7, 330.1 ± 21.2, 140.3 ± 18.6, and 90.4 ± 6.5 μm, respectively, for 48 h of incubation.

Inhibition of Lewis lung carcinoma metastasis in mice

The effect of CH1104I on pulmonary metastasis of LLC cells transplanted in C57/BL6 mice was examined. As shown in Table 1, the number of tumor nodes formed on the lung surface was significantly lower in the CH1104I-treated mice than in untreated mice ($P < 0.05$). The antimetastasis ability of CH1104I was dose-dependent. In contrast, a significant weight loss was not observed during the continuous administration (Table 1).

Fig. 7



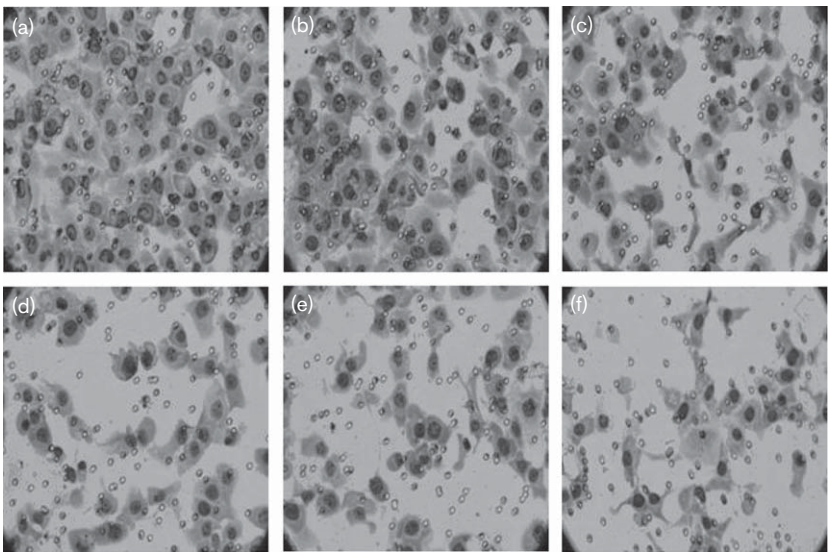
Effects of (*S*)-methyl 6-(benzyloxycarbonylamino)-2-(2-((*S*)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido) piperidin-1-yl) acetamido) hexanoate (CH1104I) on the expressions of matrix metalloproteinase (MMP)-2 and MMP-9 in SKOV3 cells were detected by western blot analysis. Cells were exposed to CH1104I (0, 7, 35, 70, 140 and 210 μmol/l) for 24 h. Cells were lysed and subjected to western blot analysis. Each of the blots shown was demonstrated to have equal proteins loading by reprobing with the monoclonal antibody for β-actin.

Discussion

In this study, we examined the effect of CH1104I, the galloyl cyclic-imide derivative, on activity and expression of MMP-2 and MMP-9 in human carcinoma cells. CH1104I significantly suppressed the activity and expression of MMP-2 and MMP-9 in SKOV3 of ovarian cells as described by gelatin zymography, immunocytochemical staining, and western blot analysis (Figs 4–7). The ability of invasion and migration of tumor cells was inhibited as a result of administration of CH1104I (Figs 8 and 9). Although CH1104I did not display a significant antiproliferative effect or cytotoxicity (Fig. 2) and the invasive potential of SKOV3 cells is dependent on the overexpression of MMP-2 and MMP-9 [11,20], the effect of antiinvasion and antimigration of CH1104I might be because of the inhibition of MMP-2 and MMP-9.

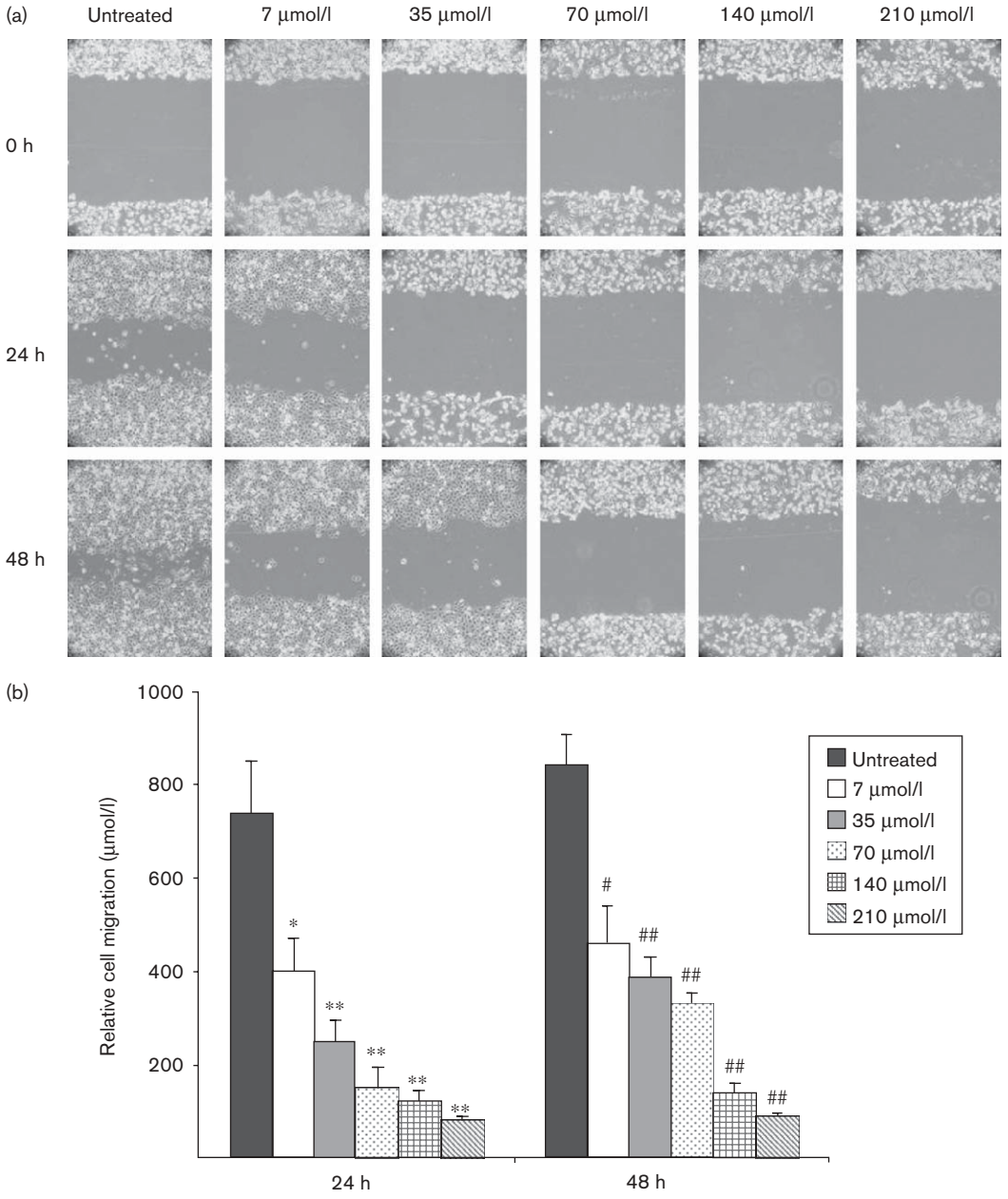
Examination of the antimetastatic ability of CH1104I *in vivo* was performed with metastatic LLC cells instead of SKOV3 cells. LLC cells overexpressing MMP-2 and MMP-9 *in vivo* are known to be more likely to develop pulmonary metastases and cellular invasiveness is known to depend on the functional consequences of increased MMP-2 and MMP-9 [21]. This study showed that oral administration of CH1104I significantly prevented the pulmonary metastasis of LLC cells in mice devoid of toxic effects. These results suggest that CH1104I may inhibit the metastasis of LLC cells by blocking the expression of MMP-2 and MMP-9.

Fig. 8



Inhibition of SKOV3 cell invasion by (*S*)-methyl 6-(benzyloxycarbonylamino)-2-(2-((*S*)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido) piperidin-1-yl) acetamido) hexanoate (CH1104I). Cells pretreated with various concentrations of CH1104I were placed on Matrigel-coated filters and incubated for 24 h. The number of cells passing through the filter was counted after staining with hematoxylin (magnification, × 400). Control (a), 7 μmol/l (b), 35 μmol/l (c), 70 μmol/l (d), 140 μmol/l (e), and 210 μmol/l (f).

Fig. 9



Inhibition of SKOV3 cell migration by (*S*)-methyl 6-(benzyloxycarbonylamino)-2-(2-((*S*)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido) piperidin-1-yl) acetamido) hexanoate (CH1104I). Scratch assay was performed by plating cells in 6-well culture dish. After cells were allowed to attach and reach 80% confluence, a scratch (1 mm) was made through culture dish with a sterile plastic 200 μl micropipette tip to generate one homogeneous wound along each well. Cells were further incubated without (control) or with different concentrations of CH1104I for 24 hand 48 h and the wound widths were measured under microscope using an ocular grid (magnification, $\times 100$). The bars indicate means ± SD ($n=3$). * and ** indicate means that are significantly different when compared with the untreated cells of 24-h incubation with *P* value of less than 0.05 and *P* value of less than 0.01, respectively. # and ## indicate means that are significantly different when compared with the untreated cells of 48-h incubation with *P* value of less than 0.05 and *P* value of less than 0.01, respectively.

Metastatic spread is a complex process involving a series of sequential events, including detachment of tumor cells from the primary site, invasion through the basement membrane and the underlying extracellular matrix (ECM) and then intravasation. Among the process of spread, MMPs' degradation of BM and ECM is a key step in the process of invasion and metastasis. Particularly, MMP-2 and MMP-9 have been found at high expression levels in most of cancers. MMP-2 and MMP-9 are synthesized and secreted as zymogens that require activation by proteolytic removal of the NH_2-terminal propeptide to convert to the active forms. Therefore,

Table 1 Effect of CH1104I on Lewis lung carcinoma metastasis in C57/BL6 mice

		Weight (g)[a]		Tumor node formation on lung surface	
Dosage (mg/kg)	Mice that survived (*n*)	Body	Lung	*n*[a]	Inhibition rate (%)[b]
CH1104I					
0	10	23.1 ± 2.3	0.20 ± 0.06	40.5 ± 6.9	-
25	9	23.6 ± 1.7	0.17 ± 0.02	23.1 ± 3.2	43.0*
50	10	22.3 ± 2.5	0.16 ± 0.07	17.6 ± 4.8	56.5*
100	10	23.1 ± 2.0	0.17 ± 0.03	13.8 ± 3.7	65.9*
Carboxylates					
100	10	22.0 ± 1.6	0.18 ± 0.08	29.0 ± 7.1	28.4*

CH1104I, (*S*)-methyl 6-(benzyloxycarbonylamino)-2-(2-((*S*)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido) piperidin-1-yl) acetamido) hexanoate.
[a]Data are indicated as the mean ± SD.
[b]Differences were analyzed using the Student's two-tailed *t*-test.
*$P < 0.05$.

regulation of MMP-2 and MMP-9 is crucial in blocking tumor invasion and metastasis. The three-dimensional structural analysis of the active sites showed that the well-defined S1′ pocket in the crystal structure of human MMP-2 and MMP-9, which facilitated catalysis, was deeper than that of other MMPs [22]. This has provided helpful clues when using structure-based design strategies to discover novel MMPs inhibitors that selectively block the proteolytic activity of MMP-2 and MMP-9.

CH1104I was designed based on L-iso-glutamine derivatives that can serve as a potential antitumor agent with low selectivity between MMPs and aminopeptidase N [8]. We modified the structures using the strategy of conformational constraint to fit the deeper S1′ active pocket of MMP-2 and MMP-9. The results showed that CH1104I significantly inhibited the activity of zymogens and the expression of active MMP-2 and MMP-9 with high selectivity [7]. As mentioned in the references [7,8,23], the L-iso-glutamine was cyclized to be a piperidinedione, which is a common structure of the cyclic-imide derivatives, hoping for exploring new metalloproteinase inhibitors with more specificity and stability. The cyclic-imide derivatives are potential MMPs inhibitors that correlate with the larger and more stable structures which accommodate to the S1′ active pocket. The carboxylic acid group of CH1104I side chains and the carbonyl group of the piperidinedione might chelate the zinc ion that is essential for enzyme activity. Although the S1′ pocket of MMP-2 and MMP-9 is deeper than that of other MMPs [22] and as the superposition of MMP-9 and MMP-2 catalytic domain structures are similar [24], we designed the backbone of cyclic-imide linked with methylated galloyl functional group to extend into the S1′ active pocket. The portion of gallic acid is also known as one of the antitumor compounds [25–27]. Gallic acid proved to be inhibitory of the activity of broad spectrum MMPs including MMP-2 and MMP-9. Besides, the three adjacent phenolic hydroxyl groups of gallic acid were methylated for more liposolubility and less cytotoxicity. The stable methylated galloyl functional group may extend into the S1′ active pocket, which may enhance the selectivity of chemical structure to combine with the active site of the specific enzymes [23,28]. These constructions in the CH1104I molecule would provide the specificity for acting on MMP-2 and MMP-9.

In conclusion, CH1104I is a novel galloyl cyclic-imide derivative with an inhibitory effect on the proteolytic activity of MMP-2 and MMP-9 in carcinoma cells. These results suggest that CH1104I may be a candidate compound for antiinvasion and antimetastasis through the suppression of MMPs activity.

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